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Characterization of the Gentamicin Resistance Transposon Tn*5281* from *Enterococcus faecalis* and Comparison to Staphylococcal Transposons Tn*4001* and Tn*4031*

SUSAN L. HODEL-CHRISTIAN AND BARBARA E. MURRAY*

Center for Infectious Diseases, Department of Microbiology, and Department of Internal Medicine, University of Texas Medical School, Houston, Texas 77030

Received 9 October 1990/Accepted 21 March 1991

In *Enterococcus faecalis*, the genetic determinant encoding gentamicin resistance (Gm^r) on the conjugative plasmid pBEM10 previously has been shown to be on a mobile element. In the current study, this element, termed Tn*5281*, was shown to relocate in the absence of homologous recombination in *E. faecalis* UV202. On the basis of restriction endonuclease analysis and DNA-DNA hybridization studies, Tn*5281* was shown to be similar, if not identical, to the Gm^r transposons Tn*4001* found in Australian isolates of *Staphylococcus aureus* and Tn*4031* found in U.S. isolates of *Staphylococcus epidermidis*, since all three of these transposons have symmetrically located *Hin*dIII (2.5 kb apart), *Cla*I (slightly more than 2.5 kb apart), and *Hae*III (3.9 kb apart) sites. Restriction endonuclease digestion patterns of Tn*5281* generated with *Hin*cII, *Sca*I, and *Alu*I were also consistent with Tn*4001* and Tn*4031*. By using a probe specific for the external portion of the terminal inverted repeat of Tn*4031*, it was determined that each terminus of Tn*5281* contained a 0.35-kb *Hae*III fragment and a 0.7-kb *Hin*dIII-*Hae*III fragment. The sizes of these fragments are identical to those found in the staphylococcal transposons, which is a further indication that inverted repeats like IS*256* are present in Tn*5281*. A 1-kb *Hae*III fragment in pBEM10 also hybridized with this probe, which indicates that Tn*5281* in pBEM10 contains a double copy of the inverted repeat at one end.

The bifunctional enzyme 6′ acetyltransferase [AAC(6′)]/2″-phosphotransferase [APH(2″)] results in resistance to gentamicin (Gm^r), tobramycin, and kanamycin in both staphylococci and enterococci (5, 6, 18, 22). The gene encoding the bifunctional enzyme is known to reside on transposons in some Australian isolates of *Staphylococcus aureus* (Tn*4001*) and some U.S. isolates of *Staphylococcus epidermidis* (Tn*4031*) and on a mobile element located on the large (70 kb), conjugative Gm^r β-lactamase-producing (Bla^+) plasmid pBEM10 present in *Enterococcus faecalis* HH22 isolated in Houston, Texas (13, 17, 19, 21). The complete nucleotide sequence of Tn*4001* has been determined, and the coding region for the bifunctional enzyme was found to be identical to that on the Gm^r plasmid pIP800 in *E. faecalis* (3, 11, 20). Both Tn*4001* and Tn*4031* have been demonstrated to move independently of homologous recombination (17, 21).

Both Tn*4001* and Tn*4031* are composite transposons 4.7 kb in length that have a 2.0-kb unique region containing the *aacA-aphD* coding region bounded on each side by 1.35-kb insertion sequences (ISs) (IS*256* in Tn*4001*) (see Fig. 4 for further details). Tn*4001* and Tn*4031* contain symmetrically located *Hin*dIII sites (2.5 kb apart) and symmetrically located *Cla*I sites (~2.5 kb apart) positioned just outside of the *Hin*dIII sites (12, 15, 21). Tn*4001* also has two *Hae*III sites located 0.35 kb apart that are positioned near the outer terminus of each IS*256* element (16); the inner *Hae*III sites are symmetrically located 3.9 kb apart (*Hae*III recognition sites have not been published for Tn*4031*). North American isolates of Gm^r *S. aureus* contain a 2.5-kb *Hin*dIII fragment that hybridizes with the 2.5-kb *Hin*dIII fragment from Tn*4001*; however, the North American isolates lack the 3.9-kb *Hae*III fragment and have symmetrically located *Bgl*II sites that generate a 3.15-kb fragment not observed in either Tn*4001* or Tn*4031* (15, 21). Despite sharing homology with the Australian isolates of *S. aureus*, Gm^r determinants in North American isolates of *S. aureus* are not mobile, since they are apparently missing part of the IS elements (11, 15, 21).

We have shown previously that the mobile element carrying the Gm^r determinant on the *E. faecalis* plasmid pBEM10 is similar to both Tn*4001* and Tn*4031*, since it has a 2.5-kb *Hin*dIII fragment containing the *aacA-aphD* coding region and does not contain any *Bgl*II sites. Also, this element was shown to insert into different locations within the conjugative, tetracycline resistance (Tet^r) plasmid, pCF10 (13). In this study, we demonstrate that this Gm^r element, now termed Tn*5281*, can move in the absence of homologous recombination (Rec^-). We also investigated the relationship between Tn*5281* and the two staphylococcal transposons (Tn*4001* and Tn*4031*) by using restriction endonuclease analysis and DNA-DNA hybridization with a Gm^r gene probe and a probe specific for the terminal inverted repeats of Tn*4031*.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and their plasmids used in this study are listed in Table 1. *Escherichia coli* SK1592(pGO121), containing Tn*4031* from *S. epidermidis*, was a gift from Gordon Archer. All bacteria were grown at 37°C. The following antibiotics were utilized as necessary: gentamicin (1,000 μg/ml), spectinomycin (1,000 μg/ml), streptomycin (2,000 μg/ml), rifampin (100 μg/ml), fusidic acid (25 μg/ml), tetracycline (10 μg/ml), and erythromycin (10 μg/ml).

DNA collection, restriction analysis, gel electrophoresis, and hybridization studies. Enterococcal plasmid DNA was col-

* Corresponding author.

TABLE 1. Bacterial strains and plasmids utilized

Strain and plasmid	Host genotype[a]	Relevant plasmid markers[b]	Plasmid size (kb)	Reference
E. faecalis				
OG1X(pBEM10::Tn*917*)	*rif fus*	Gm^r Bla^+ Em^r Tra^-	~75	13
OG1SSp(pCF10)	*str spc*	Tet^r Tra^+	58	8
JH2-2	*rif fus*			14
OG1SSp	*str spc*			9
UV202	*rif fus rec*			24
UV202(pBEM10::Tn*917*, pCF10)	*rif fus rec*	Gm^r Bla^+ Em^r Tet^r	~75, 58	This study
A [JH2-2(pCF10::Gm^r)]	*rif fus*	Tet^r Gm^r Em^s Bla^-	~63	13
B [JH2-2(pCF10::Gm^r)]	*rif fus*	Tet^r Gm^r Em^s Bla^-	~63	13
C [JH2-2(pCF10::Gm^r)]	*rif fus*	Tet^r Gm^r Em^s Bla^-	~63	This study
D [OG1SSp(pCF10::Gm^r)]	*str spc*	Tet^r Gm^r Em^s Bla^-	~63	This study
E [OG1SSp(pCF10::Gm^r)]	*str spc*	Tet^r Gm^r Em^s Bla^-	~63	This study
F [OG1SSp(pCF10::Gm^r)]	*str spc*	Tet^r Gm^r Em^s Bla^-	~63	This study
E. coli				
SF815A(pSF815)[c]		Gm^r Amp^r	4.2	11
SK1592(pGO121)[d]		Gm^r	14	21

[a] Resistance to fusidic acid (*fus*), rifampin (*rif*), streptomycin (*str*), spectinomycin (*spc*), and recombination deficient (*rec*).
[b] Abbreviations: Amp, ampicillin; Tra, transfer function.
[c] Contains *aacA-aphD* gene from the *E. faecalis* plasmid pIP800.
[d] Contains Tn*4031* from *S. epidermidis*.

lected by using a modification for gram-positive bacteria of Currier and Nester's (7) cell lysis protocol and cesium chloride-ethidium bromide density gradient centrifugation (23). A modification of the alkaline lysis technique of Birnboim and Doly (2) was used to collect plasmid DNA from *E. coli* SK1592(pGO121).

Restriction endonucleases were used as recommended by the supplier (Bethesda Research Laboratories, Inc., Gaithersburg, Md.), except that reactions were allowed to proceed for up to 5 h to ensure complete digestion of the large resistance plasmids. Fragments generated by restriction endonuclease digestion were separated in agarose gels (0.7%) by electrophoresis. The running buffer (also contained in the gel) was 1× Tris-borate buffer (10× TBE is 1 M Tris-base, 0.89 M boric acid, 0.5 mM EDTA, pH 8.0). DNA fragments were transferred from agarose gels to Hybond-N membrane filters according to the recommendation of the manufacturer (Amersham Corp., Arlington Heights, Ill.).

A probe specific for the coding region of the bifunctional enzyme (the Gm^r gene probe) was made by double digestion of pSF815A with *Eco*RI and *Hin*dIII, as described previously (13). The 1.5-kb *Eco*RI-*Hin*dIII fragment used for a probe was separated from the 2.7-kb vector fragment by agarose (1.0%) gel electrophoresis with 1× Tris-acetate buffer (10× TAE is 0.4 M Tris, 0.2 M sodium acetate, 20 mM EDTA, pH 8.0) in the gel and used as the running buffer. The DNA was extracted from the gel by using GENECLEAN (BIO 101, Inc., La Jolla, Calif.). A probe containing the portion of the IS*256*-like terminal inverted repeats exterior to the *Hin*dIII sites was made by cloning an ~1.0-kb *Hin*dIII fragment from pGO121 into pUC18 (25). This clone was double digested with *Cla*I and *Hin*dIII and the ~1-kb *Cla*I-*Hin*dIII fragment was collected from polyacrylamide (5%) gels for use as a probe (the IS*256*-like probe). The *Cla*I-*Hin*dIII fragment was about 20 bp smaller than the cloned *Hin*dIII fragment. DNA was extracted from the polyacrylamide gels by placing a gel slice that had been cut into small pieces into a microcentrifuge tube containing 1 ml of gel elution buffer (0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, 0.1% sodium dodecyl sulfate). Following overnight incubation at 37°C with shaking, polyacrylamide fragments were removed by centrifugation for 2 min and the DNA in the supernatant was precipitated by adding 2 volumes of ice-cold 95% ethanol. After incubating on dry ice for 30 min, the DNA was pelleted by spinning for 20 min in a microcentrifuge and washed with 70% ethanol. Both probes were labeled by using the Random Primed DNA Labeling kit (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) and [α-^{32}P]dCTP (Amersham). Hybridization was done under highly stringent conditions at 42°C with prehybridization and hybridization solutions used as recommended by Amersham (5× SSPE [1× SSPE is 0.18 M NaCl, 1 mM EDTA, 10 mM $NaPO_4$], 5× Denhardt solution, 0.5% sodium dodecyl sulfate, 50% formamide, and 100 μg of denatured calf thymus DNA per ml).

Method used for mating experiments. Transposition of Gm^r was detected by using cross-streak mating (1). Mating plates were incubated overnight at 37°C. Growth present in the area of the cross was resuspended in 1.0 ml of sterile saline (0.9% NaCl) and serial 10-fold dilutions were made (to 10^{-4}), if necessary. Aliquots (100 μl) were plated onto Todd-Hewitt agar plates containing the appropriate antibiotics to select for the recipient cells: tetracycline resistant (Tet^r) (indicating transfer of pCF10) and Gm^r. All Tet^r Gm^r transconjugants were checked for susceptibility to erythromycin (Em^s) and for lack of β-lactamase production.

RESULTS AND DISCUSSION

Transposition of Tn*5281* in Rec^+ *E. faecalis*. Initially, transposition of Gm^r determinants in *E. faecalis* was detected by the movement of Gm^r from pBEM10::Tn*917* (Gm^r, Em^r, Bla^+, Tra^-) into and cotransfer with the conjugative Tet^r plasmid, pCF10. These studies were done in *E. faecalis* strains having functional homologous recombination systems. We had analyzed two Tet^r, Gm^r, Em^s, and Bla^- transconjugants (plasmids A and B) previously (13), and in this study have isolated a third transconjugant with the same resistance pattern (plasmid C) by using cross-streak mating between OG1X(pBEM10::Tn*917*, pCF10) with JH2-2. The resistance profile of plasmids A, B, and C suggested that the Gm^r gene from pBEM10 had inserted into pCF10. Retransfer

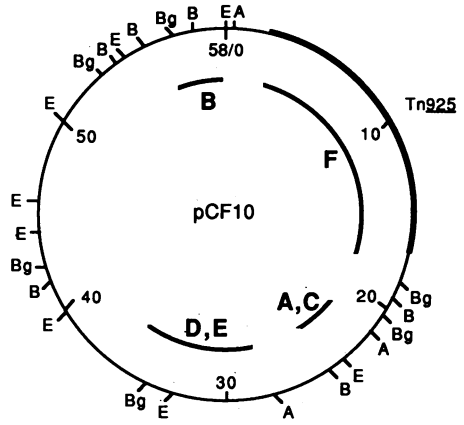

FIG. 1. Simplified restriction endonuclease map of pCF10 (based on the figure in reference 6) showing regions into which Tn*5281* inserted (plasmids A through F) (inside circle). Precise locations of insertion sites were not determined. Regions shown were determined by comparison of restriction endonuclease patterns generated for pCF10 to those obtained for plasmids A through F and determining which fragment(s) had changed in size. All 6 plasmids (A through F) are the same size and are ~4.7 kb larger than pCF10. Plasmids A, B, C, and F each result from separate insertion events. Plasmids D and E probably are the result of the same transposition event. Plasmids A and C contain the same *Eco*RI, *Bam*HI, *Bgl*II, and *Ava*I fragments; however, the insertion sites are known to be located in two different locations within those fragments on the basis of the restriction patterns generated by digestion of the plasmid DNA with *Hin*dIII and *Hae*III. Transposition events resulting in plasmids A, B, and C were observed in the presence of the homologous recombination system, while those resulting in plasmids D, E, and F were observed in the absence of homologous recombination. The transposon Tn*925* is an integral part of pCF10 and is indicated by the dark portion of the circle. Abbreviations present on the outside of the circle to indicate restriction endonuclease recognition sites of pCF10: A, *Ava*I; B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI.

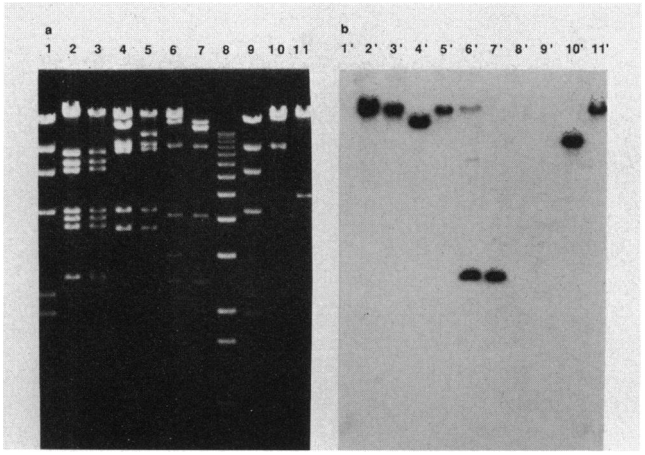

FIG. 2. (a) Agarose (0.7%) gel electrophoresis of restriction digests of plasmid DNA from plasmids C (lanes 2, 4, 6, and 10) and F (lanes 3, 5, 7, and 11) with *Eco*RI (lanes 2 and 3), *Bgl*II (lanes 4 and 5), *Cla*I (lanes 6 and 7), and *Ava*I (lanes 10 and 11). Molecular weight standards were lambda digested with *Hin*dIII (lanes 1 and 9) and a 1-kb ladder (Bethesda Research Laboratories) (lane 8). (b) Autoradiograph of filter of agarose gel in panel a after hybridization with the ^{32}P-labeled Gm^r gene probe.

of plasmid DNA from the three transconjugants (plasmids A, B, and C) to OG1SSp verified the insertion of the Gm^r determinants into pCF10. Restriction endonuclease analysis demonstrated that plasmids A, B, and C had inserted into three different positions in pCF10, with plasmids A and C being located close to each other within the same *Eco*RI and *Bam*HI fragments. *Hin*dIII and *Hae*III digests verified that plasmids A and C inserted within ~100 to 200 bp of each other. The general location of the insertion sites of Tn*5281* in pCF10 are shown in Fig. 1. The precise locations of the Tn*5281* insertion sites are not known; therefore, in Fig. 1, the sites are shown as broad regions, because of lack of known restriction sites in pCF10 available to further define the insertion locations.

Transposition of Tn*5281* in a Rec$^-$ background. To ascertain whether the Gm^r determinant could move in the absence of homologous recombination, both pCF10 and the transfer-deficient pBEM10::Tn*917* had to be transferred into UV202, a recombination-deficient *E. faecalis* strain. Since pCF10 is able to comobilize pBEM10::Tn*917* at a very low frequency, we cross-streak mated UV202 with OG1X(pBEM10::Tn*917*, pCF10) and selected fusidic acid resistant, Rif^r, Gm^r, Tet^r, Em^r, and Bla$^+$ transconjugants. This resistance profile indicated that pCF10 had comobilized pBEM10::Tn*917* and both plasmids were in UV202. One of the UV202 transconjugants was cross-streak mated with OG1SSp, and several Tet^r, Gm^r, Em^s, and Bla$^-$ transconjugants were obtained. Most transconjugants obtained were Tet^r, Gm^s, Em^s, and Bla$^-$, indicating that pCF10 does not contain a copy of Tn*5281*, and this suggests that pCF10 was not present in UV202 as a cointegrate with pBEM10::Tn*917* that resolved in a *rec*-independent fashion. Restriction analysis and DNA-DNA hybridization studies of plasmid DNA from one Tet^r, Gm^r, Em^s, and Bla$^-$ transconjugant (plasmid F) confirmed that the Gm^r determinant is carried on a transposon (designated Tn*5281*) that is able to move in a Rec$^-$ background. Figure 2 shows a comparison of DNA from plasmids C and F digested with *Eco*RI, *Bgl*II, *Cla*I, and *Ava*I. Both plasmids C and F are the same size and are approximately 5 kb larger than pCF10. The basic restriction patterns for plasmids C and F are almost identical to those obtained for pCF10, except for changes in sizes of one band or the presence of an additional band(s). Tn*5281* is present in the same *Eco*RI fragment of pCF10 in both plasmids C and F; however, Tn*5281* is in different *Bgl*II and *Ava*I fragments in these plasmids. The data indicate that Tn*5281* has inserted into a fourth location in pCF10 (Fig. 1). Patterns obtained from two other Tet^r, Gm^r, Em^s, and Bla$^-$ transconjugants (plasmids D and E) were identical to each other and revealed that Tn*5281* had inserted into a fifth location in pCF10 (Fig. 1). This fifth insertion site is in the region encoding pCF10's transfer-related genes. When a pure culture of either plasmid D or E was grown in broth, constitutive clumping of the bacterial cells occurred, and both strains yielded dry, crusty colonies on agar, which suggests that Tn*5281* had inserted into a region necessary for regulation of the aggregation factor involved in the pheromone response system of pCF10 (4, 10).

Restriction analysis and hybridization with a Gm^r probe. Gm^r transposons from *E. faecalis* (Tn*5281*) and *S. epidermidis* (Tn*4031* in pGO121) were compared by using restriction endonuclease analysis and DNA-DNA hybridization of plasmids pBEM10, A, B, C, and pGO121. Both transposons are known to contain symmetrically located *Hin*dIII sites positioned 2.5 kb apart on which *aacA-aphD* resides (13, 21).

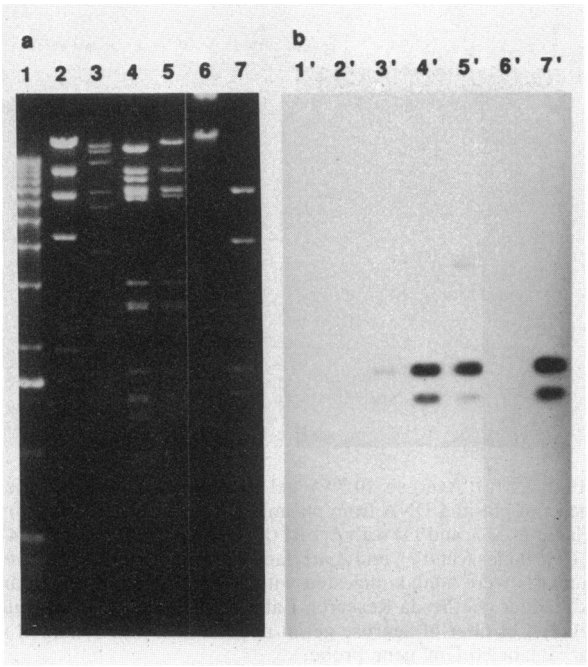

FIG. 3. (a) Agarose (0.7%) gel electrophoresis of restriction endonuclease digestions with *Sca*I (lanes 3 through 7). Plasmid DNA digested was from pBEM10 (lane 3), plasmid A (lane 4), plasmid B (lane 5), pCF10 (lane 6), and pGO121 (lane 7). Molecular weight standards were a 1-kb ladder (Bethesda Research Laboratories) (lane 1) and *Hin*dIII-digested lambda DNA (lane 2). Note: incomplete digestion of pCF10. (b) Autoradiograph of filter of agarose gel in panel a after hybridization with the ^{32}P-labeled Gm^r gene probe.

Digestion of plasmids pBEM10, A, B, C, and pGO121 with *Hae*III revealed the presence of a 3.9-kb fragment in both Tn*5281* and Tn*4031*; this fragment hybridized with the Gm^r gene probe. Since Tn*4001* of *S. aureus* is known to have symmetrically located *Hae*III sites 3.9 kb apart, our data suggest that both Tn*5281* and Tn*4031* are closely related to Tn*4001*. *Cla*I digests of the Tn*5281*-containing plasmids revealed the presence of a 2.5-kb fragment that was the same size as a fragment generated by *Cla*I digestion of Tn*4031*. Tn*4001* also is known to have symmetrically located *Cla*I sites (~2.5 kb apart) that are positioned 25 bp outside of the *Hin*dIII sites. *Cla*I digestion of plasmids C and F and their hybridization data are shown in Fig. 2 (lanes 6 and 7). Hybridization revealed that the 2.5-kb *Cla*I fragments contained the *aacA-aphD* gene.

*Bgl*II was also used to digest *E. faecalis* plasmid DNA,

TABLE 2. Summary of hybridization with the Gm^r gene probe

Plasmid or transposon	Size and restriction fragment hybridizing (kb)						
	*Hin*dIII	*Hae*III	*Bgl*II	*Cla*I	*Hin*cII	*Sca*I	*Alu*I
pBEM10	2.5	3.9	>15	2.5	1.2, 2.2	1.5, 1.8	1.5
A, B, C, and F	2.5	3.9	>15	2.5	1.2, 2.2	1.5, 1.8	1.5
pGO121[a]	2.5	3.9	7.0	2.5	1.2, 2.2	1.5, 1.8	1.5
Tn*4001*[b]	2.5	3.9	~17	2.5	1.2, 2.2	1.5, 1.8	1.5

[a] Contains Tn*4031*.
[b] Tn*4001* fragments listed as showing hybridization are deduced from the published nucleotide sequence (3, 20).

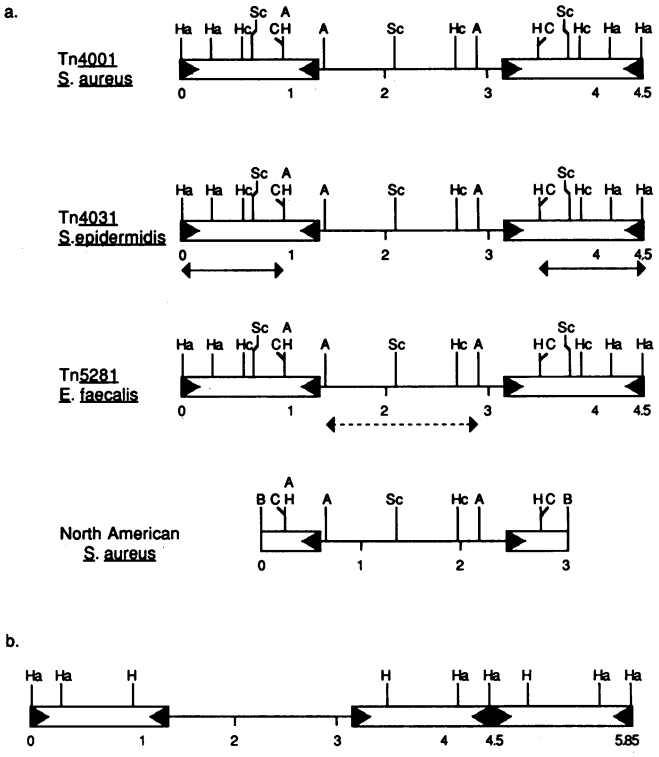

FIG. 4. (a) Restriction endonuclease maps of Tn*4001* from Australian isolates of Gm^r *S. aureus* (3, 15, 20), Tn*4031* from U.S. isolates of Gm^r *S. epidermidis* (21), the Gm^r transposon from *E. faecalis*, and the region of the Gm^r gene from North American isolates of *S. aureus*. Boxes with arrows indicate the position of terminal inverted repeats (IS*256* of Tn*4001*). Dashed line with arrows at ends indicates the region contained in the Gm^r gene probe. Solid line with arrows at ends indicates area used for the IS*256*-like probe. (b) Simplified restriction endonuclease map of Tn*5281* present in plasmids pBEM10 and B. The original copy of Tn*5281* in *E. faecalis* strain 67×22 along with the DNA from one of transposition events (plasmid B) each contain a double copy of the IS*256*-like element at one end of the transposon. Restriction endonuclease abbreviations: Ha, *Hae*III; H, *Hin*dIII; C, *Cla*I; B, *Bgl*II; Hc, *Hin*cII; Sc, *Sca*I; A, *Alu*I. Note: for clarity only relevant *Cla*I, *Hin*cII, and *Alu*I sites are shown.

since a number of North American isolates of Gm^r *S. aureus* have symmetrically located *Bgl*II sites (3.15 kb apart) encompassing the Gm^r gene which are not present in Tn*4001* or Tn*4031*. Digestion of the plasmids containing Tn*5281* revealed no 3.15-kb *Bgl*II fragments, and when hybridization was performed with the Gm^r gene probe, large fragments (>15 kb) hybridized. These results verify that Tn*5281* has more similarities to the staphylococcal Gm^r transposons Tn*4001* and Tn*4031* than to the nonmobile Gm^r determinants found in North American *S. aureus* isolates.

By using the published nucleotide sequence of Tn*4001* as a guide, three restriction endonucleases (*Hin*cII, *Sca*I, and *Alu*I) were chosen to further analyze the relatedness of the three Gm^r transposons (3, 20). All three of these restriction endonucleases are known to have one or two recognition sites in the region containing the *aacA-aphD* coding region and at least one site in each insertion sequence. On the basis of the sequence of Tn*4001*, two *Hin*cII fragments (1.2 and 2.2 kb) should hybridize with the Gm^r gene probe if the Gm^r transposons are related. This hybridization pattern was seen

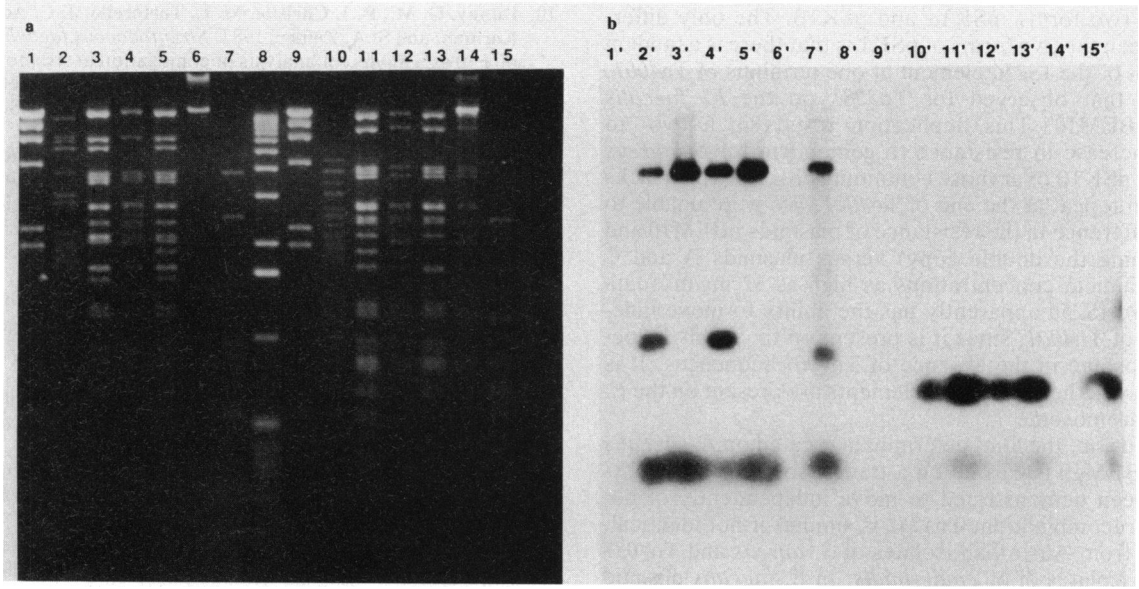

FIG. 5. (a) Agarose (0.7%) gel electrophoresis of restriction endonuclease digests with *Hae*III (lanes 2 through 7) and *Hae*III-*Hin*dIII (lanes 10 through 15) of pBEM10 (lanes 2 and 10), DNA from plasmids A (lanes 3 and 11), B (lanes 4 and 12), C (lanes 5 and 13), pCF10 (lanes 6 and 14), and pGO121 (lanes 7 and 15). Molecular weight standards were lambda DNA digested with *Hin*dIII (lanes 1 and 9) and 1-kb ladder (lane 8). Note: digestion of plasmid pCF10 was incomplete. (b) Autoradiograph of filter of agarose gel in panel a following hybridization with the ^{32}P-labeled IS*256*-like probe.

with both Tn*5281* and Tn*4031*. Similar data were obtained with *Sca*I, which showed hybridization of two fragments (1.5 and 1.8 kb), as expected (Fig. 3, lanes 3 through 7). Finally, as expected because both Tn*4001* and the enterococcal Gm^r plasmid pIP800 are known to have two *Alu*I sites located 1.5 kb apart in the unique region containing the *aacA-aphD* coding region, hybridization of a 1.5-kb *Alu*I fragment was observed. Hybridization data using the Gm^r gene probe for the enterococcal transposon, Tn*4031*, and the published data for Tn*4001* are summarized in Table 2.

Restriction maps of Gm^r transposons. By using restriction endonuclease analysis and DNA-DNA hybridization data, a restriction map was prepared for Tn*5281*. The restriction maps for Tn*4001*, Tn*4031*, Tn*5281*, and the nonmobile Gm^r determinants in North American isolates of *S. aureus* are shown in Fig. 4a. All of our data suggest that Tn*5281* is similar, if not identical, to Tn*4001* and Tn*4031* and unlike the nonmobile Gm^r determinants from North American isolates of *S. aureus* that contain a 3.15-kb *Bgl*II fragment. The nonmobile Gm^r determinants from isolates of *S. aureus* also contain the *aacA-aphD* gene and hybridize with the Gm^r probe (data not shown).

Hybridization with an IS*256*-like probe. Figure 5 shows fragments generated by digestion of plasmids pBEM10, A, B, C, pCF10, and pGO121 with *Hae*III (lanes 2 through 7) and *Hae*III-*Hin*dIII (lanes 10 through 15). Southern blots using a probe specific for the terminal inverted repeats of Tn*4031* (the IS*256*-like probe) are also shown. Since Tn*5281* is very similar to Tn*4001* and Tn*4031*, hybridization was expected to be observed with the 0.3-kb (doublet) and 3.9-kb *Hae*III fragments, plus the 0.7-kb *Hae*III-*Hin*dIII fragment (doublet). All these fragments hybridized with the probe as expected (lanes 2 through 7 and 10 through 15). However, the hybridization results suggest that plasmids pBEM10 and B (lanes 2′ and 4′) contain a double copy or tandem repeat of the IS*256*-like element at one end, since a 1.0-kb *Hae*III fragment hybridized with the IS*256*-like probe in addition to the 0.3- and 3.9-kb *Hae*III fragments. Neither plasmid A nor C contained the extra *Hae*III fragment. Both the 0.3-kb *Hae*III (doublet) and 0.7-kb *Hae*III-*Hin*dIII (doublet) fragments hybridized when the plasmid DNA was double digested with *Hae*III and *Hin*dIII (lanes 10′ through 15′). The tandem IS elements appear to be positioned in the same orientation with respect to each other, on the basis of hybridization patterns seen with *Hae*III. *Hin*dIII digestion of the plasmid DNA confirmed the orientation of the tandem duplication, since a 1.3-kb *Hin*dIII fragment hybridized with the IS*256*-like probe in both plasmids pBEM10 and B (Table 3). If the double IS elements had been in opposite orientations, a 2.0-kb *Hin*dIII fragment would have hybridized with the probe instead of the 1.3-kb *Hin*dIII fragment. Table 3 contains a summary of the hybridization results observed with the IS*256*-like probe. The hybridization data suggest that Tn*5281* in pBEM10 (original location) has a tandem repeat of the IS*256*-like element at one end and that the transposon can apparently jump with (plasmid B) or without the double IS element (plasmids A and C) (Fig. 4b).

According to Lyon et al. (16), the plasmid pSK1 on which Tn*4001* resides in Australian isolates of Gm^r *S. aureus* can be

TABLE 3. Summary of hybridization with the IS*256*-like probe

Plasmid	Size of restriction fragments hybridizing (kb)		
	*Hae*III[a]	*Hae*III-*Hin*dIII	*Hin*dIII
pBEM10	0.35, 1.0, 3.9	0.35, 0.7	1.3, 1.6, 4.8
A	0.35, 3.9	0.35, 0.7	1.8, 3.0
B	0.35, 1.0, 3.9	0.35, 0.7	1.3, 2.8, 3.1
C	0.35, 3.9	0.35, 0.7	1.9, 2.8
pGO121	0.35, 0.8, 3.9	0.35, 0.7	ND[b]

[a] All 0.35-kb fragments that hybridized appeared to be present in at least two copies.
[b] ND, not determined.

present in two forms, pSK1α and pSK1β. The only difference between the two forms of pSK1 is that there is a tandem duplication of the IS*256* element at one terminus of Tn*4001*, similar to that observed for Tn*5281* on the *E. faecalis* plasmid pBEM10. This duplication results in a two- to fourfold increase in resistance to gentamicin for *S. aureus* containing pSK1β over those containing pSK1α, which lacks the tandem repeat at the end of Tn*4001*. We were unable to detect a difference in the resistance of plasmids pBEM10 and B (containing the double copy) versus plasmids A and C using gentamicin concentrations as high as 32 mg/ml (data not shown). IS*256* apparently has the ability to move independently of Tn*4001*, since it is present on the staphylococcal chromosome in the absence of Tn*4001* sequences. It is not known whether IS*256*-like elements are present on the *E. faecalis* chromosome.

In conclusion, the Gm^r determinant located on *E. faecalis* plasmid pBEM10 is carried on a transposon termed Tn*5281* that has been demonstrated to move independently of homologous recombination. Tn*5281* is similar, if not identical, to Tn*4001* from Australian isolates of *S. aureus* and Tn*4031* from U.S. isolates of *S. epidermidis*. In *E. faecalis* plasmid pBEM10, Tn*5281* contains a double copy of the IS*256*-like element at one terminus and can apparently transpose with or without the additional copy of the IS element. Further studies are under way in an attempt to determine whether Tn*5281* would be a useful tool for transposon mutagenesis in *E. faecalis* and other gram-positive organisms.

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